DNAs OR GENES PARTICIPATING IN PARKINSON'S DISEASE

TECHNICAL FIELD

The present invention relates to a gene responsible for onset of Parkinson's disease. Since it was found that Parkinson's disease patients have deletion in part of the gene, the gene of this invention is significantly useful as a gene for diagnosing Parkinson's disease, and a protein and a pharmaceutically active agent etc., obtainable from the inventive gene has usability in preventing and treating Parkinson's disease.

BACKGROUND ART

Generally, it is often considered that one or more gene is responsible for various chronic progressive diseases. Isolating the gene or genes responsible for these diseases not only enables to facilitate prenatal or postnatal diagnosis but also enables to perform gene therapy for the disease based on the remarkable progress and development of gene therapy as seen today.

Parkinson's disease is one of chronic diseases. α-synuclein reported in 1997 has so far been only the gene that has found to be responsible for Parkinson's disease. It is

reported that some people having Italian ancestry suffer from autosomal dominant Parkinson's disease due to mutation of this gene. There is, however, limitation in diagnosing Parkinson's disease even with use of this gene. Therefore, what has been adopted at present as a diagnosis for Parkinson's disease is merely a clinical approach based on neurodegenerative symptoms such as resting tremor, rigidity, akinesia, and disturbance of the righting reflux, and a levodopa-responsive or dopaminergic compound (agonist) has been administered as a symptomatic treatment. So far no drastic therapy has been performed for treating Parkinson's disease.

DISCLOSURE OF THE INVENTION

The present invention has been made in view of the above. An object of this invention is to provide an isolated DNA or gene or gene fragment that is responsible for Parkinson's disease and is useful in diagnosing and treating the disease etc.; a recombinant vector; a protein or polypeptide; a monoclonal antibody or polyclonal antibody; a primer or probe or immobilized nucleic acid or DNA chip; and an oligonucleotide, or the like.

The isolated DNA or gene according to this invention that has overcome the above problems residing in the prior art is:

① An isolated DNA or gene: comprising a full-length base

sequence according to the sequence ID. No. 1 or 2 [the sequence ID. No. 2 does not include a base portion 636 to 719 (corresponding to exon 5 which is described later) of the sequence ID. No. 1, namely, a variant thereof according to alternative splicing], or a partial sequence thereof, or a base sequence hybridizable thereto or hybridizable with a complemental strand thereof, and being associated with Parkinson's disease.

Further, the inventive DNA or gene may include a DNA or gene or gene fragment having the following features ② to ⑧, in addition to ①.

② An isolated DNA or gene: comprising the base sequence of ①, or the full-length base sequence thereof, or the base sequence partially thereof, and the isolated DNA or gene whose gene defect is responsible for Parkinson's disease, or comprising a base sequence hybridizable thereto or hybridizable with a complemental strand thereof.

③ An isolated DNA or gene comprising the base sequence of ① or ②, the isolated DNA or gene being variant thereof by alternative splicing, and being associated with Parkinson's disease, or the isolated DNA or gene comprising a base sequence hybridizable thereto or hybridizable with a complemental strand thereof.

④ A gene comprising the base sequence of any one of ① to ③ whose gene product encodes a protein having a substantially

equivalent function to a protein comprising 1 to 465 amino acid sequence in the sequence ID. No. 1 or to a protein comprising 1 to 437 amino acid sequence in the sequence ID. No. 2.

⑤ An isolated DNA or gene comprising a gene which has caused an exonic deletion, a nonsense base substitute, a missense base substitute, a base deletion, a base addition, a base insertion, a splicing abnormality and/or a frameshift with respect to the base sequence of any one of ① to ④; or comprising a base sequence hybridizable thereto or hybridizable with a complemental strand thereof, and the isolated DNA or gene being associated with Parkinson's disease.

⑥ An isolated DNA or a gene, or a gene fragment comprising a partial base sequence of the DNA or the gene of any one of claims ① to ⑤, or an isolated DNA or a gene or a gene fragment comprising a base sequence hybridizable thereto or hybridizable with a complemental strand thereof.

⑦ A gene encoding a protein (a) or (b) comprising:

(a) the protein comprising 1 to 465 amino acid sequence in the sequence ID. No. 1;

(b) the protein in which one or more amino acid(s) of the amino acid sequence is or are deleted, substituted, or added, and the protein being associated with Parkinson's disease.

⑧ A gene encoding a protein (c) or (d):

(c) the protein comprising 1 to 437 amino acid sequence

in the sequence ID. No. 2;

(d) the protein in which one or more amino acid(s) of the amino acid sequence is or are deleted, substituted, or added, and the protein being associated with Parkinson's disease.

The full-length base sequence in the sequence ID. No. 1 is such that eleven introns are intervened among twelve exons on the genome; and encodes a protein having 1 to 465 amino acid sequence in a part (102 to 1496) of the base sequence. The base sequence of the intron in a boundary region between the exon and the intron has the following arrangement:

the intron intervening between exon 1 and exon 2 has a base sequence shown in the sequence ID. No. 3 adjacent to the 3' end of the exon 1, and has a base sequence shown in the sequence ID. No. 4 adjacent to the 5' end of the exon 2;

the intron intervening between exon 2 and exon 3 has a base sequence shown in the sequence ID. No. 5 adjacent to the 3' end of the exon 2, and has a base sequence shown in the sequence ID. No. 6 adjacent to the 5' end of the exon 3;

the intron intervening between exon 3 and exon 4 has a base sequence shown in the sequence ID. No. 7 adjacent to the 3' end of the exon 3, and has a base sequence shown in the sequence ID. No. 8 adjacent to the 5' end of the exon 4;

the intron intervening between exon 4 and exon 5 has a base sequence shown in the sequence ID. No. 9 adjacent to the

3' end of the exon 4, and has a base sequence shown in the sequence ID. No. 10 adjacent to the 5' end of the exon 5;

the intron intervening between exon 5 and exon 6 has a base sequence shown in the sequence ID. No. 11 adjacent to the 3' end of the exon 5, and has a base sequence shown in the sequence ID. No. 12 adjacent to the 5' end of the exon 6;

the intron intervening between exon 6 and exon 7 has a base sequence shown in the sequence ID. No. 13 adjacent to the 3' end of the exon 6, and has a base sequence shown in the sequence ID. No. 14 adjacent to the 5' end of the exon 7;

the intron intervening between exon 7 and exon 8 has a base sequence shown in the sequence ID. No. 15 adjacent to the 3' end of the exon 7, and has a base sequence shown in the sequence ID. No. 16 adjacent to the 5' end of the exon 8;

the intron intervening between exon 8 and exon 9 has a base sequence shown in the sequence ID. No. 17 adjacent to the 3' end of the exon 8, and has a base sequence shown in the sequence ID. No. 18 adjacent to the 5' end of the exon 9;

the intron intervening between exon 9 and exon 10 has a base sequence shown in the sequence ID. No. 19 adjacent to the 3' end of the exon 9, and has a base sequence shown in the sequence ID. No. 20 adjacent to the 5' end of the exon 10;

the intron intervening between exon 10 and exon 11 has a base sequence shown in the sequence ID. No. 21 adjacent to the 3' end of the exon 10, and has a base sequence shown in the

sequence ID. No. 22 adjacent to the 5' end of the exon 11; and

the intron intervening between exon 11 and exon 12 has a base sequence shown in the sequence ID. No. 23 adjacent to the 3' end of the exon 11, and has a base sequence shown in the sequence ID. No. 24 adjacent to the 5' end of the exon 12.

In addition, a recombinant vector comprising the DNA fragment or the gene of any one of ① to ⑧ may be included in the scope of this invention.

The protein which has overcome the above problem is (i) a protein comprising 1 to 465 amino acid sequence in the sequence ID. No. 1; or (ii) a protein comprising 1 to 437 amino acid sequence in the sequence ID. No. 2, the protein being associated with Parkinson's disease; or a protein having a substantially equivalent function thereto.

More specifically, the protein or polypeptide according to this invention may embrace the following aspects (ii) to (viii).

(ii) A protein expressed by the gene of any one of ① to ④, the protein being associated with Parkinson's disease, or having an identical function thereto or a substantially equivalent function thereto.

(iii) A protein comprising an amino acid sequence translated by the gene of ⑤, and the protein being associated with Parkinson's disease, or having an identical function thereto or a substantially equivalent function thereto.

(iv) A protein comprising the amino acid sequence of (iii) in which an amino acid is substituted, deleted, or added at least at one position, and the protein being associated with Parkinson's disease.

(v) A protein comprising the amino acid sequence of any one of (ii) to (iv) comprising: a ubiquitin-like 1 to 72 amino acid sequence partially included in the sequence ID. No. 1; and a zinc-finger-protein-like 418 to 449 amino acid sequence partially included in the sequence ID. No. 1.

(vi) A protein (a) or (b):

(a) the protein comprising 1 to 465 amino acid sequence in the sequence ID. No. 1;

(b) the protein in which one or more amino acid(s) of the amino acid sequence is or are deleted, substituted, or added, and the protein being associated with Parkinson's disease.

(vii) A protein (c) or (d):

(c) the protein comprising 1 to 437 amino acid sequence in the sequence ID. No. 2;

(d) the protein in which one or more amino acid(s) of the amino acid sequence is or are deleted, substituted, or added, and the protein being associated with Parkinson's disease.

(viii) A polypeptide or a protein consisting of a partial fragment of the amino acid sequence of any one of (i)

(vii), or comprising the partial fragment thereof, or the full-length amino acid sequence thereof.

In addition, a monoclonal antibody or a polyclonal antibody against the protein of any one of (i) to (viii) may be included in the scope of this invention.

Further, a primer, or a probe, or an immobilized nucleic acid, or a DNA chip according to this invention may preferably be used for the following purposes (I) to (IV):

(I) for use in detecting a base sequence, a genetic mutation, a deletion, and/or an expression amount of the DNA or the gene of any one of ① to ⑧, or for use in concentration thereof;

(II) for use in detecting a base sequence, a genetic mutation, a deletion, and/or an expression amount of RNA which is subjected to transcription and subjected to processing from the DNA or the gene of any one of ① to ⑧, or for use in concentration thereof;

(III) for use in detecting a base sequence, a genetic mutation, and/or a deletion of the exon in the sequence ID. No. 1 or No. 2, or for use in haplotyping a locus thereof; or

(IV) for use in detecting a base sequence, a genetic mutation, and/or a deletion of the aforementioned intron, or for use in haplotyping a locus thereof.

Specifically, at least one of fourteen set of primers or probes shown in the following (1) to (14) can be used.

(1) A primer or a probe for use in detecting a base sequence of the intron adjacent to the exon 1 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or probe comprising the following base sequence:

a base sequence of the sequence ID. No. 25 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 26 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(2) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 2 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 27 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 28 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(3) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 3 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 29 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 30 in the 5'-3'

direction on a complemental strand of the sequence ID. No. 1 on the genome.

(4) A primer or a probe for use in detecting a base sequence of the intron adjacent to the exon 4 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or probe comprising the following base sequence:

a base sequence of the sequence ID. No. 31 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 32 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(5) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 4 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 33 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 34 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(6) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 5 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 35 in the 5'-3'

direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 36 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(7) A primer or a probe for use in detecting a base sequence of the intron adjacent to the exon 6 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or probe comprising the following base sequence:

a base sequence of the sequence ID. No. 37 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 38 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(8) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 7 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 39 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 40 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(9) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 7 of the gene being associated with Parkinson's disease of ①, or a locus thereof,

the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 41 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 42 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(10) A primer or a probe for use in detecting a base sequence of the intron adjacent to the exon 8 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or probe comprising the following base sequence:

a base sequence of the sequence ID. No. 43 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 44 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(11) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 9 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 45 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 46 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(12) A primer or a probe for use in detecting a base

sequence of an intron adjacent to the exon 10 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 47 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 48 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(13) A primer or a probe for use in detecting a base sequence of the intron adjacent to the exon 11 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or probe comprising the following base sequence:

a base sequence of the sequence ID. No. 49 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 50 in the 5'-3' direction on a complemental strand of the sequence ID. No. 1 on the genome.

(14) A primer or a probe for use in detecting a base sequence of an intron adjacent to the exon 12 of the gene being associated with Parkinson's disease of ①, or a locus thereof, the primer or the probe comprising the following base sequence:

a base sequence of the sequence ID. No. 51 in the 5'-3' direction of the sequence ID. No. 1 on the genome, and

a base sequence of the sequence ID. No. 52 in the 5'-3'

direction on a complemental strand of the sequence ID. No. 1 on the genome.

Further, the present invention may include the following oligonucleotide, or an oligonucleotide analog, or a modified product thereof as shown in (a) to (c):

(a) the one which comprises a partial sequence of the base sequence of any one of ① to ⑧, or which is hybridizable with the base sequence of any one of ① to ⑧.

(b) the one for use in amplifying the full-length base sequence or the partial base sequence of any one of ① to ⑧, or the oligonucleotide for use in amplifying partially the full-length base sequence or the partial base sequence of any one of ① to ⑧, according to PCR method using a human RNA as a template, PCR method or RT-PCR method using a human cDNA as a template.

(c) the oligonucleotide for use in amplifying the base sequence comprising the exon in the sequence ID. No. 1 or No. 2 and the aforementioned intron which is adjacent to the exon according to PCR method, or the oligonucleotide for use in amplifying a part of the base sequence according to PCR method.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram showing the pedigree of family members including Parkinson's disease patient in Example 1.

FIG. 2 is a diagram showing results of haplotyping

according to PCR analysis with respect to the family members including Parkinson's disease patient in FIG. 1.

FIG. 3 is a diagram showing presence or absence of gene deletion in the family members including Parkinson's disease patient in FIG. 1.

FIG. 4 is a diagram showing an alignment of six cDNA fragments and one full-length gene isolated in Example 3.

FIG. 5 is a diagram showing amino acid sequence homology between the N-terminus of this inventive gene and ubiquitin.

FIG. 6 is a diagram showing the result of gel electrophoresis of EcoRI digest of genomic DNA fragments with respect to the family members including Parkinson's disease patient in FIG. 1.

FIG. 7 is a diagram showing the pedigree of family members including Parkinson's disease patients in Example 7.

FIG. 8 is a diagram showing presence of absence of gene deletion with respect to the family members including Parkinson's disease patient in FIG. 7.

FIG. 9 is a diagram showing cDNA fragment of the Parkinson's disease patient in FIG. 7, obtained by Example 7.

FIG. 10 is diagram showing mRNAs of the inventive gene that is expressed in various human tissues.

FIG. 11 is diagrams showing mRNAs of the inventive gene that is expressed in various human tissues.

FIG. 12 is a diagram showing the pedigree of family

members including Parkinson's disease patients in Example 9.

FIG. 13 is a diagram showing presence or absence of gene deletion in the family members including Parkinson's disease patient in FIG. 12.

FIG. 14 is a diagram showing the result of gel electrophoresis of EcoRI digest of genomic DNA fragments with respect to the family members including Parkinson's disease patient in FIG. 12.

FIG. 15 is a diagram showing the pedigree of family members including Parkinson's disease patients in Example 10.

FIG. 16 is a diagram showing presence or absence of gene deletion with respect to the family members including Parkinson's disease patient in FIG. 15.

FIG. 17 is a diagram showing the pedigree of family members including Parkinson's disease patients in Example 11.

FIG. 18 is a diagram showing presence or absence of gene deletion with respect to the family members in FIG. 17.

FIG. 19 is a diagram showing the pedigree of family members including Parkinson's disease patients in Example 12.

FIG. 20 is a diagram showing presence or absence of gene deletion with respect to the family members including Parkinson's disease patient in FIG. 19.

FIG. 21 is a diagram showing the pedigree of family members including Parkinson's disease patients in Example 13.

FIG. 22 is a diagram showing presence or absence of gene

deletion with respect to the family members including Parkinson's disease patient in FIG. 21.

FIG. 23 is a diagram showing the pedigree of family members including Parkinson's disease patient in Example 14.

FIG. 24 is a chromatogram showing the result of direct sequencing of PCR products from exon 5 of a wild allele and a mutant allele.

FIG. 25 is a diagram showing DNA and amino acid sequences of a wild-type (W) parkin gene, and predicted sequences of a mutant (M) parkin molecule having one-base deletion.

FIG. 26 is a diagram showing the result of NlaIV restriction site analysis of PCR products in Example 14.

FIG. 27 is photomicrographs showing the result of immunohistochemical staining of the inventive gene that is expressed in brain sections.

FIG. 28 is photomicrographs showing the result of immunostaining of the inventive gene that is expressed in brain sections, polyubiquitin, and α-synuclein.

FIG. 29 is a diagram showing the result of immunoblotting the whole homogenates of the frontal lobe of control subjects, Parkinson's disease patients, and AR-JP patients.

FIG. 30 is a diagram showing the result of immunoblotting the subcellular fractions of the frontal lobe

tissue of a control subject.

FIG. 31 is a diagram showing the result of immunoblotting the whole homogenates of the SN, putamen, and frontal lobe of control subjects, Parkinson's disease patients, and AR-JP patient.

FIG. 32 is a diagram showing the result of gel electrophoresis of PCR products from exon 4 and exon 10.

FIG. 33 is a graph obtained by hydropathy plot of the amino acid sequence of this inventive protein.

FIG. 34 is a diagram showing that replacement of -366 Arg to Trp changes α helix portion to β-sheet structure.

BEST MODE FOR CARRYING OUT THE INVENTION

Parkinson's disease or Parkinsonism is considered to be initiated by genetic predisposition and environmental factors. Elucidating individual factors is an urgent matter on fundamental understanding and treatment of Parkinson's disease and Parkinsonism in their onset stage. The inventors of this invention have studied to find out a gene responsible for onset of Parkinson's disease. As a result of their study, it was found that the region of chromosome 6q25.2-q27, more specifically, a 17-cM region between two chromosome markers DS437 and D6S264 has a strong linkage with juvenile Parkinsonism, which is one of Parkinson's disease (Matsumine et al., Am. J. Hum. Genet. 60(1997)588-596). The inventors have

succeeded in isolating the gene responsible for Parkinson's disease by conducting the below-mentioned Examples with respect to the juvenile Parkinson's disease patients and thus accomplished the present invention.

Hereinafter, the present invention is described in detail with reference to the process of experiments that have contributed to the finding of the inventive gene. It should be appreciated that the following examples are illustrative and not restrictive, and all changes that fall within metes and bounds of the claims, or equivalence of such metes and bounds are therefore intended to be embraced by the claims.

Example 1: Chromosomal Deletion Region in Juvenile Parkinson's disease Patient

FIG. 1 shows the pedigree of family members including Parkinson's disease patient in Example 1. In FIG. 1, an open square represents an unaffected male, an open circle represents an unaffected female, and a filled circle represents the affected female. The circle or square with slash represents the deceased member. Although the parents and brothers of the patient are not affected, the patient had Parkinson-like symptom from her teens and diagnosed as Parkinson's disease. The symptom has been gradually progressing.

Haplotyping according to PCR method was performed using D6S305 which is one of the markers of chromosome 6 with respect to the genomic DNA of subjects marked with an asterisk in FIG.

1 (patient and two unaffected members). The result is shown in FIG. 2.

As shown in FIG. 2, D6S305 was amplified from DNA template of the parents and brother of the patient, however, D6S305 was not amplified from DNA template of the patient. It was verified accordingly that the patient has deletion of D6S305 which is one of the chromosomal markers.

Example 2: Screening of Genomic Fragment Including D6S305 and Exon Trapping

Since Example 1 verified that the patient has deletion of the genomic DNA corresponding to marker D6S305, there is a possibility that a gene responsible for Parkinson's disease may exist on the genomic DNA. To verify the possibility, PCR screening was performed to isolate normal human genomic library consisting of 96,000 genomic fragment (the Keio human BAC library) using a set of amplimers having a sequence of part of marker D6S305. As a result of screening, two clones, genimic fragments KB761D4 and KB430C4 each of which has an insert size of about 110kb were isolated.

Next, exon trapping was performed to isolate exon fragment of the gene existing on the genomic fragments using exon trapping system (provided by GIBCO/BRL) according to the manufacturer's instruction manual. As a result, the isolated exon was J-17 only despite the fact that the two genomic fragments each had a relatively large size of about 110kb.

Subsequently, base sequence of the intron adjacent to exon J-17 was determined based on the base sequence of exon J-17 by using a PCR primer to amplify exon J-17 itself (J-17 Inner), and BAC KB761D4 as template. Then, two sets of PCR primer (J-17 outer) were prepared to amplify the fragment including J-17 based on the thus-determined sequence of the intron. In this way, PCR amplification analysis was performed for the genomic DNA of the subjects shown in Example 1 (patient and the unaffected parents and brother). The result of analysis is shown in FIG. 3. It should be noted that FIG. 3 shows the result of Example 6 as well as the result of Example 2.

As shown in FIG. 3, no PCR product was detected from the genomic DNA of the patient (lane 3) whereas PCR product was detected from the normal genomic DNA of the father (lane 1), mother (lane 2) and brother (lane 4). This suggests that the patient has at least chromosomal deletion corresponding to exon J-17 of the inventive gene.

Example 3: Screening of Inventive Gene from Normal Human cDNA Library

Next, screening of the inventive gene was performed using human cDNA library to isolate cDNAs which cover the full-length of the gene including J-17 together with full-length of its translation sequnence. Specifically, cDNA libraries of normal human fetal brain and skeletal muscle were purchased

from Clontech. J-17 fragment, which is part of exon of the inventive gene and was isolated in Example 2, was used as an initial probe, and insertion DNA fragment of positive clones isolated by initial screening using the initial probe were used as probes for secondary screening. As a result, seven cDNA clones [HFB1, HFB3, HFB4, HFB5, SKM1, SKM3, and SKM8] shown in FIG. 4 were isolated. The insertion DNA fragments of positive clones were amplified with two set of vector-specific primer (F10inner: 5'-AGCCTGGTTAAGTCCAAGCTG-3' and R10inner: 5'-GAAGGTCCCATTTTTCGTTTTC-3').

The thus amplified positive DNA fragment was sequneced directly according to primer walking method. Cycle sequencing was performed using the above-mentioned primers and a commercial kit [ABI PRISM labeling kits (manufactured by Perkin-Elmer)] and ABI model 377DNA sequencer (manufactured by Applied Biosystems) according to the manufacture's instruction manual.

As a result, it was found that seven cDNAs had an piled relationship as shown in FIG. 4. The longest base sequence SKM8 has 2960bp which includes a full-length of translation sequence which encodes a protein containing 1395bp (nt102 to nt1496 or to nt1499 including stop codon), and 465 amino acids.

Also, it was found that four cDNA clones (HFB3, HFB4, SKM1, and SKM3) of seven cDNA lost 84bp from nt636 to nt719. This implicates that there are at least two ways of splicing

when mature mRNA grows from this inventive gene on genome by splicing.

Further, it was verified that the N-terminal portion (ranging from methionine-1 to arginine-72) of the protein consisting of 1 to 465 amino acid sequence which is encoded by the inventive gene has a moderate homology (content of the same amino acid: 33%) with ubiquitin as shown in Fig.5.

Ubiquitin is known as a significant substance which removes a protein that has no longer been necessary in a cell, and involvement with various neurodegenerative diseases has also been pointed out. For instance, it has been known that paired helical filaments (PHFs) in Alzheimer's disease and Lewy bodies in Parkinson's disease are stained by an anti-polyubiquitin antibody. The mechanism is considered to act as follows. Ubiquitin is conjugated with various proteins and forms multi-ubiquitin chain by repeated conjugations, and induces to inhibit proteasome pathway, finally to be metabolized.

Lysine residue-48 is known to be an essential element for ubiquitin-conjugate. Since lysine exists at position 48 in the above protein, and the amino acid sequence at the vicinity of the target region (for instance, positions 44 to 48 and position 51) conforms with that of ubiquitin, it is suggested that the above protein has ubiquitin-like function. Further, recent studies found some of conjugated proteins contain

ubiquitin-like portion at the N-terminal portion thereof. The latter finding implies that the ubiquitin-like portion acts as a molecular chaperone.

Although homology with ubiquitin is observed at the N-terminal portion of this inventive protein as described above, homology with ubiquitin is seldom observed with respect to amino acid sequence at position 73 and thereafter. As another feature, the protein of this invention has amino acid sequence at the vicinity of the C-terminal portion (positions 418 to 449) containing a large number of cysteine residues: Cys-X_2-Cys-X_9-Cys-X_1-His-X_2-Cys-X_4-Cys-X_4-Cys-X_2-Cys. This sequence is extremely similar to that of a ring-finger motif (Cys-X_2-Cys-$X_{(9-399)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys), a kind of sequence of a zinc-binding motif in a zinc-finger protein (a protein conjugated with zinc, and deeply involved in growth, differentiation, and generation of a cell). Accordingly, it is presumed that the protein of this invention is one of novel zinc-finger proteins.

Example 4: Screening of the inventive Genomic Gene from Genome Library

As mentioned above, only exon (J-17) was found in two positive genomic clones (KB761D4 and KB430C4) which were obtained in Example 2. In this Example, by the purpose of obtaining a genimic fragment containing other exon(s), BAC clone screening was performed by hybridization of DNA from a

genome library consisting of 95,232 clones (Keio human BAC library), using SKM8 clone which has the largest size among the positive clones obtained from the aforementioned cDNA library, as a probe. As a result, 24 new positive clones were obtained.

In addition, a PCR primer for amplifying exon 1, which corresponds to the N-terminal portion of the cNDA base sequence was prepared, screening of BAC library according to PCR amplification was performed, and another new positive clone was obtained.

Identification of each exon and sequencing of intron adjacent to each exon were performed according to Primer walking method (BEE procedure) using the above-obtained twenty five clones. As a result of analysis, exon 1 to 3, 5, 6, and 8-12 were mapped to either one of the twenty five BAC clones. Also, it was verified that J-17 corresponds to exon 7. BAC clones having genomic sequence including exon 4 were not, however, found in the twenty five BAC clones.

Another PCR primer to amplify exon 4 was prepared, and two new positive clones were obtained by PCR screening using a genome library supplied by Genome Systems Inc. Sequencing of each exon and intron adjacent to each exon was performed according to the aforementioned primer walking method with use of twenty seven BAC-DNA clones as template. The primers used in primer walking method were appropriately prepared based on cDNA sequence. BAC clones corresponding to the respective

primers were separated according to oligonucleotide colony hybridization using primer itself as a probe. DNA sequencer was used for their sequencing.

As a result, the alignment of exon and intron of this inventive gene was made clear. It was verified that the gene of this invention has a very large spanning over 500kb and consists of twelve exons intervening very large eleven introns. The intron sequence in the boundary region between exon and intron was described as above. Table 1 shows the whole base sequences in exon-intron boundaries.

Table 1

Intron - exon boundaries of Parkin gene

	Exon	Intron	Exon	
Exon 1	ACCATGATAG	gtacgtgggt....ccttggtcag	TGTTTGTCAG	Exon 2
Exon 2	GACTGTGCAG	gtgagtctcc....tcccaaacag	AATTGTGACC	Exon 3
Exon 3	GGAAGTCCAG	gtaattggaa....tcttctccag	CAGGTAGATC	Exon 4
Exon 4	CTTGACCCAG	gtaaggaaat....tttcccaaag	GGTCCATCTT	Exon 5
Exon 5	GACTAGTGCA	gtaagtacct....tttctttcag	GAATTTTTCT	Exon 6
Exon 6	CAGACGTCAG	gtaaggatct....ctctctgcag	GAGCCCCGTC	Exon 7
Exon 7	CCTTGTGTGG	gtaagtctag....tttccaacag	CTGGCTGTCC	Exon 8
Exon 8	AGAAGAGCAG	gtgagtgagc....ggttttgcag	TACAACCGGT	Exon 9
Exon 9	GGGCTGTGGG	gtgagtactg....tcttttgcag	TTTGCCTTCT	Exon 10
Exon 10	AACTACTCAG	gtacagaatg....gtttccccag	GCCTACAGAG	Exon 11
Exon 11	GAAAAAAATG	gtgagtctgt....cccccaacag	GAGGCTGCAT	Exon 12

Example 5: Determination of Base Sequence of Exonic Part on Genomic DNA

Next, the base sequence of exon part obtainedd in Example 4 was amplified to verify whether the base sequence of the part conforms with that of the corresponding part of cDNA. Specifically, fourteen sets of primers were prepared based on flanking intron sequence at the 5'-terminus and 3'-terminus of each exon (part of the primer was prepared based on partial sequence of exon), and PCR amplification was performed with use of DNAs which have been prepared by the standard procedure with use of normal human peripheral blood leukocytes as template. For reference, Table 2 shows the base sequences of the fourteen primer sets used in this Example.

Table 2

Primer sequences and sizes of expected PCR products

	Exon	primer	Forward (5'- 3')	Reverse (5'- 3')	product size(bp)
1	1	Ex 1	GCGCGGCTGGCGCCGCTGCGCGCA	GCGGCGCAGAGAGGCTGTAC	112
2	2	Ex 2	ATGTTGCTATCACCATTTAAGGG	AGATTGGCAGCGCAGGCGGCATG	308
3	3	Ex 3	ACATGTCACTTTTGCTTCCCT	AGGCCATGCTCCATGCAGACTGC	427
4	4	Ex 4 Inner	AGGTAGATCAATCTACAACAGCT	CTGGGTCAAGGTGAGCGTTGCCTGC	121
5	4	Ex 4 outer	ACAAGCTTTTAAAGAGTTTCTTGT	AGGCAATGTGTTAGTACACA	261
6	5	Ex 5	ACATGTCTTAAGGAGTACATTT	TCTCTAATTTCCTGGCAAACAGTG	227
7	6	Ex 6	AGAGATTGTTTACTGTGGAAACA	GAGTGATGCTATTTTTAGATCCT	268
8	7	J-17 Inner	GAGCCCCGTCCTGGTTTTCC	CCACACAAGGCAGGGAGTAGCCAA	137
9	7	J-17 outer	TGCCTTTCCACACTGACAGGTACT	TCTGTTCTTCATTAGCATTAGAGA	239
10	8	Ex 8	TGATAGTCATAACTGTGTGTAAG	ACTGTCTCATTAGCGTCTATCTT	206
11	9	Ex 9	GGGTGAAATTTGCAGTCAGT	AATATAATCCCAGCCCATGTGCA	278
12	10	Ex 10	ATTGCCAAATGCAACCTAATGTC	TTGGAGGAATGAGTAGGGCATT	165
13	11	Ex 11	ACAGGGAACATAAACTCTGATCC	CAACACACCAGGCACCTTCAGA	303
14	12	Ex 12	GTTTGGGAATGCGTGTTTT	AGAATTAGAAAATGAAGGTAGACA	255

The above PCR amplification was carried out in the following manner. In this Example, 10 ml-reactions were prepared, each of which contained 100 ng DNA, 1×PCR buffer [50 mM Tris-HCl (pH 9.2 at 25℃), 14 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$], 350 μM each dNTP, 0.5 μM each primer and 0.35 U Expand Long Taq polymerase (Boerhinger Manheim). PCR conditions were at 94℃ for 30 sec., 50-53℃ for 30 sec., 68℃ for 30 sec. to 1 min. and repeated 35 cycles.

Base sequence of each DNA fragment that has been amplified by the above PCR was determined using appropriate PCR primers and a commercial kit. The result of sequencing is shown in Table 2.

As shown in Table 2, it was verified that the base sequence amplified according to the PCR using the aforementioned primers conforms with that of the corresponding part of cDNA.

Example 6: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 1)

In this Example, abnormality of the inventive gene was examined using the juvenile Parkinson's disease patient and family members in Example 1.

Specifically, genomic DNAs were prepared from the leukocytes of the subjects, and PCR amplification was carried out using the genomic DNAs as template and primer sets consisting of forward (5'-3') and reverse (5'-3') of exon 2,

exon 3, J-17 inner, J-17 outer, and exon 8 among the primer sets listed in Table 2. The result of analysis is shown in FIG. 3.

As seen from FIG. 3, in the case where the genomic DNAs of father (lane 1), mother (lane 2), and brother (lane 4) of the Parkinson's disease patient were used as template, the sequence corresponding to each exon was amplified. This result verifies that the genomic DNAs of these family members do not have deletion or significant mutation. On the other hand, in the case where the genomic DNA of the Parkinson's disease patient (lane 3) was used as template, no amplification of the base sequence of the genomic DNA corresponding to exons 3, 4, 5, 6, 7 was found. This result clarified that the genomic gene of the patient has a deletion of long base sequence corresponding to exons 3 to 7.

Furthermore, the genomic DNAs of the subjects were digested with EcoRI, electrophoresed, and blotted onto nylon membrane by Southern blot analysis, and P-labeling of SKM8 cDNA probe was performed by Southern blot hybridization. As a result, as shown in FIG 6., whereas at least eight EcoRI fragments were found in the parents and brother of the patient, only four fragments were found in the patient (in FIG. 6, asterisk denotes the four EcoRI fragment that has not been detected in the patient). This result also verifies that the genomic gene of the patient has deletion or mutation at a

certain part thereof.

Example 7: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 2)

As can be seen from Example 6, it is obvious that juvenile Parkinson's disease patients have deletion or the like in the inventive gene. The above example strongly implicates that deletion or the like of the inventive gene is responsible for juvenile Parkinson's disease. To further verify this, similar experiments were conducted with respect to another unrelated family members to those in Example 6.

Specifically, genome analysis was carried out with respect to the family members including juvenile Parkinson's disease patients of the pedigree in FIG. 7. Whereas two siblings out of six are unaffected, the other four siblings are all juvenile Parkinson's disease patients. PCR analysis was performed in accordance with the procedure in Example 5 using primers corresponding to respective exons with use of the genomic DNAs of the members marked with asterisk (namely, unaffected mother, two unaffected brothers, and two affected sisters) as template. The result of analysis is shown in FIG. 8.

As can be seen from FIG. 8, whereas both of the genomic DNAs of the two patients (lanes 2 and 3) in this Example show deletion of exon 4, none of the genomic DNAs of the other subjects (unaffected mother (lane 1) and two unaffected sisters

(lane 5 and 6)) have deletion.

Furthermore, mRNA was extracted from the brain tissue of one of the patients according to the standard AGPC procedure. Total 1 mg of mRNA was primed at 50℃ for 30 min. using Titan™ one tube RT-PCR System kit (Boehringer Manheim), and the reaction mixture was directly used for PCR with forward primer (nt 351 to nt 371 of Sequence ID No. 1) 5'-GGAGGCGACGACCCCAGAAAC-3' and reverse primer (nt 963 to nt 983 of Sequence ID. No. 1) 5'-GGGACAGCCAGCCACACAAGG-3'. PCR was performed at 94℃ for 30 sec., 56℃ for 30 sec., 68℃ for 1 min. and repeated 45 cycles. cDNA sequence of PCR products obtained by the above procedure was analyzed, and the result of analysis is shown in FIG. 9.

As seen from FIG. 9, mRNA of the patient shows complete deletion of exon 4, exon 3 is contiguous to exon 5 directly by skipping exon 4. Consequently, it was verified that the juvenile Parkinson's disease patients of two unrelated family members have deletion of exon in the inventive gene and that deletion of the inventive gene is responsible for juvenile Parkinson's disease.

Example 8: Inventive Gene mRNA Expression in Various Tissues

In this Example, Northern blot analysis was carried out using the genomic fragment J-17 in order to examine how widely mRNA [Poly(A)$^+$] of the inventive gene is expressed in

various human tissues.

Specifically, Northern blots of various human tissues were purchased from Clontech, and northern blotting was carried out according to the provided instruction manual with use of J-17 corresponding to exon 4 of the inventive gene, as a probe. The result of analysis is shown in FIGs. 10 to 11. It should be noted that tissues in FIG. 10A are, from left to right in the order, are spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte; those in FIG. 10B are, from left to right in the order, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas; those in FIG. 10C are, from left to right in the order, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, and bone marrow; those in FIG. 11A are, from left to right in the order, cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe, and putamen; and those in FIG. 11B are, from left to right in the order, amygdala, caudate nucleus, corpus callosum, hippocampus, whole brain, substantia nigra, subthalamic nucleus, and thalamus.

The results of FIGs. 10 to 11 show that mRNA was particularly richly expressed in the tissue of brain, heart, testis and skeletal muscle although mRNA of 4.5kb including poly A-tail was detected in all the tissues examined in this Example. It was further verified that expression was

particularly remarkable in the cerebral cortex and frontal lobe although the expression was detected in every section of the brain.

Example 9: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 3)

FIG. 12 shows the pedigree of family members including Parkinson's disease patients in Example 9. In this Example, genomic DNAs were prepared from leukocytes of the subjects marked with numerals 1 to 7 in FIG. 12 (namely, unaffected parents, three unaffected sisters, and two affected brothers) and used as template. PCR amplification was performed using oligonucleotide primer pairs shown in Table 3.

Table 3

Primer sequences and sizes of PCR products

Exon	primer	Forward (5'- 3')	Reverse (5'- 3')	product size(bp)
1	Ex 1	GCGCGGCTGGCGCCGCTGCGCGCA	GCGGCGCAGAGAGGCTGTAC	112
2	Ex 2	ATGTTGCTATCACCATTTAAGGG	AGATTGGCAGCGCAGGCGGCATG	308
3	Ex 3	ACATGTCACTTTTGCTTCCCT	AGGCCATGCTCCATGCAGACTGC	427
4	Ex 4	ACAAGCTTTTAAAGAGTTTCTTGT	AGGCAATGTGTTAGTACACA	261
5	Ex 5	ACATGTCTTAAGGAGTACATTT	TCTCTAATTTCCTGGCAAACAGTG	227
6	Ex 6	AGAGATTGTTTACTGTGGAAACA	GAGTGATGCTATTTTTAGATCCT	268
7	Ex 7	TGCCTTTCCACACTGACAGGTACT	TCTGTTCTTCATTAGCATTAGAGA	239
8	Ex 8	TGATAGTCATAACTGTGTGTAAG	ACTGTCTCATTAGCGTCTATCTT	206
9	Ex 9	GGGTGAAATTTGCAGTCAGT	AATATAATCCCAGCCCATGTGCA	278
10	Ex10	ATTGCCAAATGCAACCTMTGTC	TTGGAGGAATGAGTAGGGCATT	165
11	Ex11	ACAGGGAACATAAACTCTGATCC	CAACACACCAGGCACCTTCAGA	303
12	Ex12	GTTTGGGAATGCGTGTTTT	AGAATTAGAAAATGAAGGTAGACA	255

All the reactions were carried out according to the following procedure. Prepared was a 25 μ l reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.02% gelatin with primers, 10 nmol of each dNTP, and 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems Division). Initial denaturation at 94℃ for 10 min. was followed by 40 cycles of 94℃ for 30 sec., 55℃ for 30 sec., and 72℃ for 45 sec., and then a final extension at 72℃ for 10 min. The PCR products were visualized on ethidium bromide-stained 2% agarose gels and the presence or absence of the target exon(s) was detected. The result is shown in FIG. 13.

As seen in FIG. 13, in the case where DNA of the two Parkinson's disease patients (lanes 6, 7) was used as template, no amplification of the regions corresponding to exon 5 was detected.

A further experiment was carried out. The genomic DNA of the Parkinson's disease patient (marked with numeral 6 in FIG. 12) and of his father was digested with EcoRI, electrophoresed, and blotted onto nylon membrane by Southern blotting. Then, P-labeling of SKM8 DNA probe was subjected to Southern blot hybridization. The result of this analysis is shown in FIG. 14. As can be seen from FIG. 14, whereas at least eight EcoRI fragments were detected in the father, only seven EcoRI fragments were detected in the patient (in FIG.

14, asterisk mark denotes the undetected EcoRI fragment). This analysis verifies that the genomic gene of the Parkinson's disease patient has deletion or mutation in a specific regions of the inventive gene.

Example 10: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 4)

Another experiment was carried out in accordance with the procedure in Example 9 except that unrelated another family members to those in Example 9 were examined. Specifically, genomic analysis was carried out for the family members including juvenile Parkinson's disease patients of the pedigree shown in FIG. 15. Four sisters out of seven are unaffected, but the other three sisters have juvenile Parkinson's disease. PCR analysis was performed in accordance with the procedure in Example 9 using primers corresponding to respective exons with use of the genomic DNAs of the subjects marked with numerals 1 to 6 in FIG. 15, as template. The result of analysis is shown in FIG. 16.

As shown in FIG. 16, in the case where the DNAs of the Parkinson's disease patients (lane 3 and 6) were used as template, no amplification was found with respect to the base sequence of the regions corresponding to exon 3.

Example 11: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 5)

Another experiment was performed in accordance with the

procedure in Example 9 except that unrelated family members to those in above Examples were examined. In this Example, genomic analysis was performed for the family members including juvenile Parkinson's disease patients of the pedigree shown in FIG. 17. Three siblings out of five are unaffected, but the other two siblings are juvenile Parkinson's disease patients. PCR analysis was performed in accordance with the procedure in Example 9 using primers corresponding to respective exons with use of the genomic DNAs of the subjects marked with numerals 1 to 3 in FIG. 17, as template. The result of analysis is shown in FIG. 18.

As shown in FIG. 18, in the case where the DNA of the Parkinson's disease patient (lane 1) was used as template, no amplification was observed with respect to the base sequence of the regions corresponding to exon 4.

Example 12: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 6)

A further experiment was conducted in accordance with the procedure in Example 9 except that unrelated family members to those in above Examples were examined. Specifically, genomic analysis was performed for the family members including juvenile Parkinson's disease patients of the pedigree shown in FIG. 19. Parents and six siblings out of eight are unaffected, but the other two siblings are juvenile Parkinson's disease patients. PCR analysis was

performed in accordance with the procedure in Example 9 using primers corresponding to respective exons with use of the genomic DNAs of the subjects marked with numerals 1 to 8 in FIG. 19, as template. The result of analysis is shown in FIG. 20.

As shown in FIG. 20, in the case where the DNA of the Parkinson's disease patients (lane 4 and 8) were used as template, no amplification was observed with respect to the base sequence of the DNAs corresponding to exon 3 and exon 4.

Example 13: Partial Deletion of Inventive Gene in Juvenile Parkinson's disease Patient (Case 7)

Another experiment was conducted in accordance with the procedure in Example 9 except that unrelated family members to those in the above Examples were examined. Specifically, genomic analysis was performed for the family members including juvenile Parkinson's disease patients of the pedigree shown in FIG. 21. Parents and one brother out of five siblings are unaffected, but the other four siblings are juvenile Parkinson's disease patients. PCR analysis was performed in accordance with the procedure in Example 9 using primers corresponding to respective exons with use of the genomic DNAs of the subjects marked with numerals 1 to 6 in FIG. 21, as template. The result of analysis is shown in FIG. 22.

As shown in FIG. 22, in the case where the DNA of the

Parkinson's disease patients (lane 2 to 6) were used as template, no amplification was observed with respect to the base sequence of the regions corresponding to exon 5.

Example 14: Identification of Homozygous One-base Deletion in Exon 5

Screening was performed to determine deletion, insertion or point mutation according to direct sequencing PCR for one patient each from the family members of pedigree shown in FIG. 23 etc. PCR was performed with chimera primers that were specific to oligonucleotide primer sequences and had the sequences of the standard sequencing primers (M13 universal and reverse primers) at their 5'-ends, respectively. Excess primers and dNTPs were removed from the PCR products with an Ultrafree-MC centrifugal filter (Millipore). The purified PCR products were sequenced by the deoxy chain termination method with an Applied Biosystems 373A DNA sequencer.

As a result of screening, one-base deletion in exon 5 was identified among the patients (see FIG. 24). In FIG. 24, the upper section (N) represents the result of direct sequencing of the PCR products from exon 5 of a wild allele, and the lower section (M) represents the result of direct sequencing of the PCR products from exon 5 of a mutant allele.

More specifically, the one-base deletion removed one guanosine from the sequence -GGT- (codon 179), causing a

frameshift that resulted in an intermediate stop codon at amino acid position 187. The nucleotide and predicted amino acid sequences are shown in FIG. 25. In FIG. 25, "Normal" section shows DNA and amino acid sequences of a wild-type allele, and "Mutant" section shows DNA and amino acid sequences of a mutant allele with one-base deletion, respectively. This one-base deletion was not detected in the normal subjects.

Next, to verify the one-base deletion in the patient (marked with numeral 3) of the pedigree shown in FIG. 23 and to identify the genotypes of her parents (marked with numerals 1 and 2) and her unaffected sister (marked with numeral 4), NlaIV restriction site analysis was performed. In this analysis, exon 5 of the subjects was amplified by primer pairs in accordance with the aforementioned procedure, and their PCR products were digested with NlaIV (New England Biolabs Inc., Massachusetts). The PCR products were electrophoresed on 3% (2% Agarose/1% NuSieve Agarose) gel and visualized with ethidium bromide. The result is shown in FIG. 26. In FIG. 26, lane 1 shows the sequence of father, lane 2 shows that of mother, lane 3 shows that of the patient, and lane 4 shows that of the unaffected sister, respectively.

The wild-type allele can be detected as an NlaIV site in exon 5, and digestion with NlaIV produced two fragments (159bp and 68bp). On the other hand, the mutant allele having

one-base deletion showed a single fragment of 227bp. This restriction site analysis verified that the patient is mutant homozygote due to one-base deletion in exon 5 whereas her parents are wild-type heterozygotes, and her unaffected sister is wild-type homozygote. These results are consistent with the mode of autosomal recessive mode of inheritance.

Example 15: Immunohistochemical and Immunofluorescence Analysis of Inventive Gene in Juvenile Parkinson's disease Patients

In order to elucidate the molecular mechanism of substantia nigra (SN) caused by mutation of the inventive gene (hereinafter, sometimes referred to as "Parkin"), localization of the protein of this invention in the brains of patients with autosomal recessive juvenile Parkinsonism (AR-JP), sporadic Parkinson's disease (PD) and normal control subjects was examined by using antibodies against the protein of this invention.

More specifically, cases of fifteen PD patients, three AR-JP patients, and eight control subjects were studied. Among AR-JP patients, case 1 and case 2 are sisters, and they had a deletion of exon 4 in the inventive gene, resulting in a truncated protein of 143 amino acids due to a stop codon generated by the frameshift 6 amino acids after codon 138. Case 3 of AR-JP patient was a 52 year-old female patient and she had a deletion of exon 3 which causes a premature

termination at amino acid 96 due to the frameshift after amino acid 58.

Two kinds of rabbit polyclonal antibodies (M-73 and M-74), rabbit polyclonal antibody against α -synuclein, and mouse monoclonal antibody against polyubiquitin were used respectively in this Example.

First of all, immunohistochemical staining was conducted according to the following procedure. Formalin-fixed paraffin-embedded sections of the midbrain, frontal lobe cortex, and putamen of the subjects were treated with anti-Parkin M-74, anti- α -synuclein, or anti-polyubiquitin antibodies after appropriate dilution by a standard avidin-biotin complex method using 3',3'-diaminobenzidine for visualization. Double-immunofluorescence was performed with rabbit anti-Parkin antibody M74 and mouse anti-polyubiquitine monoclonal antibody, and subsequent incubation with FITC-conjugated goat anti-rabbit IgG (Dako, Carpinteria, CA), biotinylated goat anti-mouse IgG (Sigma, St. Louis, Mo) and avidin-rhodamine (Sigma). Signal was observed under a fluorescent confocal microscope MRC-1024 (Bio-Rad, Richmond, CA). The results of observation are shown in FIGs. 27 and 28.

FIG. 27 are photomicrographs of immunohistochemical staining with anti-Parkin antibody M-74 in the brain sections, wherein 27A to 27C show the result of a PD patient (case 2), 27D to 27F show that of a control subject (case 1), and 27G

to 27I show that of a AR-JP patient (case 1). More specifically, the photomicrographs 27A, 27D, 27G show the melanin-containing neurons in the SN, 27B, 27E, 27H show the putamen, and 27C, 27F, 27I show the frontal lobe cortex. In the photomicrographs, the point of arrow indicates neuron, the root thereof indicates neuromelanin, and the unit length of bar thereof is 50μm.

As can be seen from FIG. 27, melanin-containing neurons in the SN (including locus coeruleus) were most intensely stained in the PD patient and the control subject, but not in the AR-JP patient (FIGs. 27A, 27D, 27G). Further, in these melanin-containing neurons of the SN, cytoplasm and granular structure as well as neuronal processes were homogeneously stained. In contrast, no staining was seen in the nuclei. Some weak staining was observed in glial cells (FIGs. 27A to 27F). Neurons in the putamen and frontal lobe cortex from the PD patient and the control subject were weakly stained in the cytoplasm and perinuclear structures (FIGs. 27B, 27C, 27E, 27F).

FIG. 28 is photomicrographs of immunohistochemical staining with this inventive gene polyubiquitin, and α-synuclein in the brain sections, wherein 28A to 28C show the melanin-containing neuron in the SN of a PD patient (case 1) double-stained with anti-Parkin antibody M74 (green: A) and monoclonal anti-polyubiquitin antibody (red: B), 28D to 28F

show midbrain cross-sections from the PD patient (case 1) stained with anti-α-synuclein antibody, and 28G to 28I show midbrain cross-sections from the PD patient (case 1) stained with anti-Parkin antibody M-74. In the photomicrographs of FIG. 28, the root of arrow indicates Lewy body, and the unit length of bar thereof is 50μm.

As a result of double immunofluorescence, the anti-Parkin and anti-polyubiquitin antibodies in the Lewy body of melanin-containing neurons of the SN were stained (FIGs. 28A to 28C). As a result of immunostaining of cross-sections of midbrain, co-localization of this inventive gene and α-synuclein in some of Lewy bodies of the PD patient (FIGs. 28D, 28E, 28G, 28H) was observed. No such staining was observed in the brain tissues of the AR-JP patients (data not shown).

The above observation results verify that whereas the protein of this invention was observed in the brains of the sporadic PD patients and control subjects, this protein was not observed in the brains of the AR-JP patients. Also, the protein of this invention was found in Lewy body of the PD patients.

Example 16: Immunoblotting of Inventive Gene in Juvenile Parkinson's disease patients

Followed by Example 15, in this Example, immunoblotting was carried out with respect to the inventive gene existing in the brains of AR-JP patients, PD patients and control

subjects with use of antibodies against the protein of this invention.

Specifically, tissue blocks of frontal lobe cortex, substantia nigra and putamen of the subjects were homogenized with a Potter-Elvehjem homogenizer in an isotonic sucrose solution (10mM Tris-HCl pH 7.4, 0.32M sucrose, 1mM Zn-acetate, 15 μg/ml leupeptin, 5 μg/ml p-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF) and 50ng/ml pepstatin). The homogenate was processed for 4 step-differential centrifugation to obtain the following fractions; Nuclear fraction (pellets after 600 $\times$ g for 10 min.), mitochondrial fraction (pellets after 7,000 $\times$ g for 10 min.), microsomal fraction (pellets after 100,000 $\times$ g for 1 hr.) and cytosolic fraction (supernatant after 900,000 $\times$ g for 1 hr.). The 900,000 $\times$ g pellet was resuspended in TES buffer containing 0.25 M sucrose and layered over a step-gradient of sucrose (0.25M, 0.86M and 1.3M) and centrifuged in an SW28 rotor at 28,000 rpm for 1 hr. at 4℃. After lipid on the top layer was aspirated, the interface between 0.5M and 0.86M sucrose layers was collected as the Golgi fraction.

Proteins in these various fractions were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane blots (Bio-Rad). The blots were soaked in Tris-buffered saline containing 0.05% Tween 20 and 5% bovine serum albumin (10mM

Tris-HCl, pH 7.6 and 150mM NaCl) at 52℃ for 1hr., probed with various antibodies such as anti-Parkin antibody M-74 in the blocking solution at 4℃ overnight, then washed with Tris-buffered saline containing 0.05% Tween 20. Anti-β-tubulin antibody (Amersham Life Science, Arlington, IL) was used as an internal control and anti-γ-adaptin antibody (Sigma) was used as a Golgi marker. Finally, blots were treated with peroxidase-conjugated goat anti-rabbit IgG (Dako) and anti-mouse IgG (Dako) at a room temperature for 1 hr. Then the reaction products were visualized using a chemi-luminescence reagent (Amersham, Buckinghamshire, UK).

The results of immunoblotting are shown in FIGs. 29 to 31. FIGs. 29 to 31 are microphotographs showing the results of immunoblotting the protein of this invention in various homogenates. FIG. 29 shows the result of whole homogenates of the frontal lobe of three control subjects (cases 1 to 3), three PD patients (cases 1 to 3) and two AR-JP patients (cases 1 and 2), wherein the left side gels are size markers, and β-tubulin is an internal marker. FIG. 30 shows the result of subcellular fractions of the frontal lobe tissue of the control subject (case 1) wherein nuclear, mitochondria, microsome, cytosol and Golgi are shown from left to right in the order, and γ-adaptin is a Golgi marker. FIG. 31 shows the result of whole homogenates of the SN, putamen and frontal lobe of the two control subjects (cases 1 and 2) and

the two PD patients (cases 2 and 3).

As a result, the protein of this invention (in FIGs denoted as "Parkin") of 52 kDa was detected in the whole homogenates of the frontal lobe cortex from the PD patients and the control subjects but not in those of the AR-JP patients (see FIG. 29). A second protein band of 41 kDa, possibly a processed form of Parkin protein, was found in the PD patients. Similar results were obtained using another antibody M-73 (data not shown).

After subcellular fractionation of the frontal lobe cortex homogenates of the control subject, majority of the inventive protein was found in the cytosol and Golgi fractions, and a minute amount of the inventive protein was found in the microsomal fraction (see FIG. 30).

Further, immunoblotting analysis of the homogenates of the SN, putamen and frontal lobe cortex from the control subjects and PD patients revealed that the inventive protein is more abundant in the SN as compared to the other parts of the brain (see FIG. 31). The inventive protein in the SN Of the Parkinson's disease patients was obviously reduced in agreement with the loss of nigral neurons in the PD patients.

The above results verified that the protein of this invention was not detected in any brain section of the AR-JP patients and that this protein exists in the melanin-containing neurons in the SN.

Example 17: Polymorphism of the Parkin Gene in Sporadic Parkinson's disease (PD) Patients and Control Subjects

In this Example, polymorphism frequency in PD was investigated. Specifically, hereditary polymorphism was analyzed according to the following procedure with respect to the subjects consisting of 160 PD patients and 160 control subjects without neurodegenerative disorders. In this Example, patients with the age of onset below 40 years old were excluded. The average age of onset was 55.4 ± 10.7. None of the PD patients had family history of PD, nor diurnal fluctuations of symptoms. The age of the control subjects was from 40 to 98 years old.

Human genomic DNA was extracted from the peripheral leukocytes of the subjects. Samples were either used immediately or stored at -20℃ until analyzed. Exons 4 and 10 of the Parkin gene were amplified by PCR using two primer pairs (exon 4: forward primer, 5'-acaagcttttaaagagtttcttgt-3', reverse primer, 5'-aggcaatgtgttagtacaca-3', exon 10: forward primer, 5'-attgccaaatgcaacctaatgtc-3', reverse primer, 5'-ttggaggaatgagtagggcatt-3').

Polymorphism that replaces Ser at amino acid position 167 to Asn (S167N) (replacement of G to A) was found in exon 4. Polymorphisms that replace Arg at amino acid position 366 to Trp (R366W) (replacement of C to T) and Val at amino position 380 to Leu (V380L) (replacement of G to C)

respectively were found in exon 10. Alleles of the polymorphisms S167N, R366W, and V380L were respectively identified by digestion with AlwNI, NciI, BsP1286I. Whereas both the S167N and R366W wild alleles created restriction sites for AlwNI and NciI, respectively, the V380L mutant allele created a restriction site for Bsp 1286I, thus identifying wild allele and mutant allele.

More specifically, the PCR products were electrophoresed on 3% agarose gel and then visualized with ethidium bromide. As a result, as shown in FIG 32, a band spanning 50bp/131bp was found by digestion with AlwNI [see FIG. 32 A)], a band spanning 68bp/97bp was found by digestion with NciI [see FIG. 32 B)], and a band spanning 57bp/108bp was found by digestion with Bsp1286I [see FIG. 32 C)].

More specifically, the PCR conditions for exon 4 were as follows. The initial denaturation was performed at 94℃ for 10 min., followed by 40 cycles of denaturation at 94℃ for 30 sec., annealing at 53℃ for 1 min, and extension at 72℃ for 1 min., with a final extension at 72℃ for 10 min.. The PCR conditions for exon 10 were as follows. The initial denaturation was performed at 94℃ for 10 min., followed by 40 cycles of denaturation at 94℃ for 30 sec., annealing at 55℃ for 30 sec., and extension at 72℃ for 45 sec., with a final extension at 72℃ for 10 min..

Subsequently, frequencies of wild-type homozygotes

(w/w), wild/mutant heterozygotes (w/m), and mutant homozygotes (m/m) were examined.

Tables 4 and 5 show the wild allele and genotype frequencies of the polymorphism S167N. Expected values in Table 5 were calculated according to the Hardy-Weinberg equilibrium.

Table 4

	Control (%)	PD (%)	Total (%)
No. of Subjects	160	160	320
No. of Chromosomes	320	320	640
Allele G	180 (56.3%)	181 (56.6%)	361 (56.4%)
Allele A	140 (43.7%)	139 (43.4%)	279 (43.6%)
χ^2 = 0.006, d.f. = 0.936[a]			

Remarks [a]: No significant difference in allele frequencies between the PD patients and the control.

Note: Expected values were calculated according to the Hardy-Weinberg equilibrium.

Table 5

	Control (%)		PD (%)		Total (%)
	Observed	Expected	Observed	Expected	
GG	58 (36.3%)	51	59 (36.9%)	51	117(36.6%)
GA	64 (40.0%)	79	63 (39.4%)	79	127 (39.7%)
AA	38 (23.7%)	30	38 (23.7%)	30	76 (23.7%)
χ^2 = 2.97, d.f.=2, p = 0.227[b] χ^2 = 3.33, d.f.=2, p = 0.189[c] χ^2 = 0.016, d.f.= 2, p = 0.992[d]					

Remarks:

[b] : The expected frequencies versus observed frequencies of the genotype in the control.

[c] : The expected versus observed frequencies of the genotype in the PD patients.

[d] : No significant difference in the genotype distribution between the PD patients and the controls.

The above results show that there is no significant difference between the PD patients and the control subjects with respect to allele and genotype frequency. Further, the frequencies of both -167Ser homozygote and -167Ser/Asn heterozygotes did not differ significantly between the two groups.

Further, Table 5 shows that the observed frequencies of

three genotypes did not significantly differ between the expected frequencies of the control subjects and those of the patients. Computer analysis verified that this replacement did not cause changes in the secondary structure of the gene products.

Table 6 shows the allele and genotype frequencies of the polymorphism V380L.

Table 6

	Control (%)	PD (%)	Total (%)
No. of Subjects	160	160	320
No. of Chromosomes	320	320	640
Allele G	309 (96.6%)	314 (98.1%)	623 (97.3%)
Allele C	11 (3.4%)	6 (1.9%)	17 (2.7%)
$\chi^2 = 1.51$, d.f. = 1, $p = 0.219^a$			

Remarks [a]: No significant difference in allele frequencies between the PD patients and the controls.

As shown in Table 6, there was no significant difference in allele frequencies of the polymorphism V380L between the PD patients and the controls. Further, it was verified that the observed frequencies of the polymorphism V380L conformed with the expected frequencies and that the

secondary structure of the gene product did not change due to this polymorphic mutation.

Next, Table 7 shows the allele and genotype frequencies of the polymorphism R366W.

Table 7

	Control (%)	PD (%)	Total (%)
No. of Subjects	160	160	320
No. of Chromosomes	320	320	640
Allele C	306 (95.6%)	316 (98.8%)	622 (97.2%)
Allele T	14 (4.4%)	4 (1.2%)	18 (2.8%)
$\chi^2 = 5.72$, d.f. =1, p = 0.017[a]			

Remarks [a]: Significant difference in allele frequencies between the PD patients and the control subjects.
(Fisher's exact probability test, p = 0.014<0.02,
Odds ratio = 3.60, 95%Cl: 0.45-6.50).

Regarding the polymorphism R366W, the expected frequencies of the three genotypes were exactly same between the PD patients and the control subjects. However, the allele frequency of R366W differed significantly between the PD patients and the control subjects. Specifically, while the allele frequency in the PD patients was 1.2%, that in the

control subjects was 4.4%. This result shows that the allele frequency in the PD patients significantly lowered compared to that in the control subjects. The odd ratio (possession ratio of allele of control subjects to PD patients) was 3.60.

FIG. 33 is a graph representing hydropathy index of amino acid sequence in the polymorphism R366W. (+) and (-) regions in FIG. 33 represents hydrophobic and hydrophilic regions, respectively. The point shown by the arrow in FIG. 33 indicates the change from hydrophilicity to hydrophobicity that is caused by replacement of -366 Arg with Trp.

FIG. 34 is a diagram showing a secondary structural change in the polymorphism R366W. This diagram shows that replacement of -366 Arg with Trp changes the α-helix (at position 361 to 376) to the β-sheet structure (at position 360-360).

This Example shows that although S167N and V380L among the three polymorphisms did not crucially influence the gene product of the PD patients, the allele frequency of polymorphism R366W was extremely low in the PD patients, and the odd ratio was calculated as 3.60. This result suggests that the allele constitute a protective factor against PD which inhibits onset of PD.

EXPLOITATION IN INDUSTRY

The invention of this application has the above-

mentioned arrangements described in terms of various aspects. The aforementioned various examples and descriptions not only identified the gene responsible for Parkinson's disease but also clarified that partial deletion or mutation etc., of the gene of this invention induces Parkinson's disease. Accordingly, onset of Parkinson's disease is easily judged by detecting the presence or absence of abnormality of the inventive gene, which is very useful in diagnosing Parkinson's disease at an initial or early stage thereof.

So-called "gene therapy" for treating Parkinson's disease patients with use of the inventive gene is also possible. Further, recombinant protein obtainable from the inventive gene is useful as a drug for preventing and/or treating Parkinson's disease. Antibody (monoclonal antibody and polyclonal antibody) against such a recombinant protein can be used for diagnosis etc., of Parkinson's disease. Utilizing such a recombinant protein enables to synthesize a pharmaceutically effective agent that is significantly useful in preventing, treating, and diagnosing Parkinson's disease etc. Thus the present invention possesses significant usability in industry because the present invention can contribute to development of various gene therapies and pharmaceutical compositions effective in various diseases focusing on Parkinson's disease and Parkinson-related diseases.